# STUDIES ON THE BIOCHEMISTRY OF EPIDERMIS

## II. Some Characteristics of Deoxyribonucleases I and II of Albino Guinea Pig Epidermis and Saline Extracts of Hair*

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In order to study the effect of beta radiation on nucleases of albino guinea pig epidermis, it was first necessary to characterize these enzymes in normal tissue. Although the guinea pig is used extensively in research, there are no reports describing the activity of its epidermal nucleases. We observed that considerable deoxyribonuclease (DNase) and ribonuclease (RNase) can be washed from the guinea pig skin surface or readily leached from excised epidermis or clipped hair (1), while others showed that RNase is present on the human skin surface (2, 3). This communication deals with some characteristics of epidermal DNase I (pH optimum, about 7.0 and requiring $Mg^{++}$ for activity) and DNase II (optimum pH, about 5.0, not requiring $Mg^{++}$) and their extracellular and intracellular concentrations (4).

### METHODS

*1. Removal of Epidermis:* The epidermis of female albino guinea pigs, weighing 500–900 grams, was removed by peeling and scraping (5).

*2. Epidermal Fractions:* Prior to homogenization, 100–500 mg of excised epidermis were washed and centrifuged at 4° C in several changes of 0.25 M sucrose (6). The pooled supernatant (final volume, 2–4 ml) contains the enzymatic activity of the interstitial compartment and/or that leached from the anuclear dead cells of the *stratum corneum*.

The washed tissue was then homogenized in a conical, all glass homogenizer (Kontes Glass, Vineland, N. J.) with 20–30 mg powdered quartz in 0.25 M sucrose at 4° C, to yield a milky 10% homogenate. The enzymatic activity in this fraction is considered as intracellular or bound to the epidermal cell.

*3. Hair Extracts:* Clipped guinea pig hair was pooled and minced with a scissor to lengths of 3 to 5 mm and 0.75 gram placed in the barrel of a 10 ml hypodermic syringe. The needle end was stoppered and 2.5 ml of 0.9% saline were added and mixed well with the hair. After 24 hours' storage at 4° C, the saline extract was expressed from the hair by inserting the plunger and applying pressure.

*4. DNase Assay:* DNase I was assayed as described (1). The solution (0.8 ml final volume), consisting of 1 mg highly polymerized salmon sperm DNA (California Foundation), 0.12 M Tris buffer, pH 7.0, 0.006 M $MgSO_4$ and 0.1 ml enzyme, was incubated for 30 minutes at 37° C. A large orifice pipette (Bellco Glass Co., Vineland, N. J.) was used to transfer homogenate aliquots. In assays for DNase II, 0.12 M acetate buffer, pH 5.0, was used; 0.006 M EDTA replaced $MgSO_4$.

Reactions were stopped by the addition of 1 ml of 1 N HCl (7). The 10 × 75 mm tubes were stored 20 to 30 minutes at 4° C and then centrifuged 30 minutes at 10,000 × g. Clear supernatant, 0.1 or 0.3 ml, was diluted to 3 ml with water and the UV absorption read at 260 mμ with the Beckman DU. "Blanks" were subtracted from the reaction flask readings and the difference recorded (Δ260). Duplicate values varied within 15%.

At first, tissue and substrate blanks were incubated with each reaction tube; however, since DNA incubated in buffer showed no significant hydrolysis during the short interval, in subsequent assays the substrate was added after the addition of acid to the tissue blank tube. There was no significant increase in acid-soluble material in the tissue blank at pH 5.0 after 30 minutes' incubation; at pH 7.0, however, a significant increase occurred due to rapid autolysis of epidermal RNA (8). A unit of DNase activity is defined as an increase of 0.10 optical density (O.D.) at 260 mμ (Δ260), 1:30 dilution. From Fig. 1, where increases in Δ260 readings with increasing enzyme concentrations are compared with those for acid-soluble organic phosphate, we note that one Δ260 unit is equivalent to 21 μg of DNA-P released. DNA hydrolysis is proportional to enzyme concentration up to about 0.20 O.D. or 2 units. The increases in O.D. are linear for about 60 minutes.

*5. Analytical Procedures:* DNA was determined by the indole method (9) after extracting the nucleic acids from a 0.1 ml aliquot of epidermal homogenate with hot 5% trichloroacetic acid (TCA).

*Protein:* After the extraction of nucleic acids, the residue was analyzed for protein by the Lowry procedure (10). There was no significant difference between protein determinations performed after

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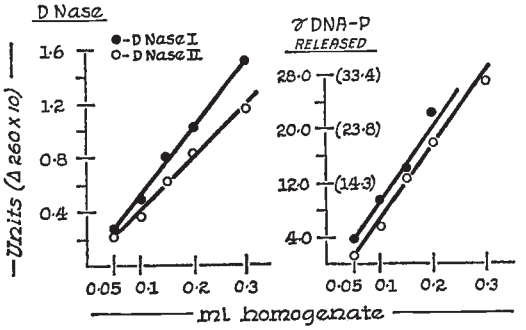


FIG. 1. Linear increases in arbitrary units (see METHODS) with increasing enzyme concentrations compared with values for acid-soluble DNA-P released. One Δ260 unit is equivalent to 21 μg DNA-P released. Figures in parentheses indicate percent of total DNA-P released as acid-soluble phosphorus.

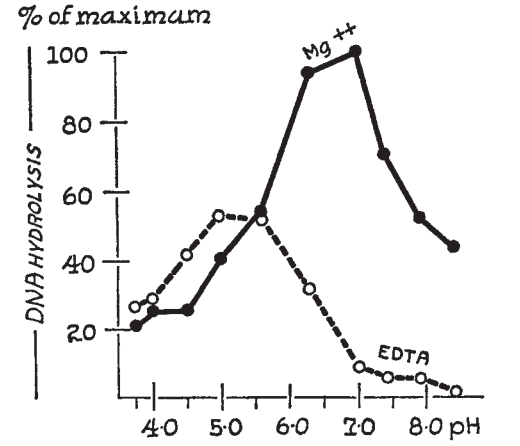


FIG. 2. pH activity curves of DNase I (solid line) and DNase II (broken line) of homogenates of washed guinea pig epidermis. Each point is the average of data from 3 animals.

extraction with either cold or hot TCA. About 15% of total epidermal protein was removed during the washing with 0.25 M sucrose (see above).

*Phosphorus:* Appropriate aliquots of the acid-soluble fraction after enzymatic hydrolysis were digested at 130° C for 1–3 hours with 0.3–0.5 ml of 10 N $H_2SO_4$. The digest was brought to 3 ml with deionized water and boiled for 10 minutes (11). Inorganic phosphorus (DNA-P) was determined by the Marsh method (12). This procedure is rapid and about 10 times more sensitive than the methods commonly in use (Fig. 1).

## RESULTS

### *Distinguishing Characteristics of Epidermal DNase I and DNase II*

*Effect of pH:* Fig. 2 shows the pH optima of epidermal DNase I and DNase II in homogenates of washed tissue. In the presence of 0.006 M $Mg^{++}$, DNase I has an optimum ranging from 6.5 to 7.0 and that for DNase II (EDTA curve) ranges from 5.0 to 5.5. Initial and final pH values were checked with the Coleman probe electrode; they varied within 0.1 unit. pH activity curves for DNase in saline extracts of hair and in washings from the skin surface obtained by the plastic cup technic (1) are shown in Fig. 3. DNase I activity predominates in both extracts; only trace amounts of DNase II are present.

Of several buffers tested, Tris gave maximal activity for DNase I and 0.12 M acetate for DNase II. The ABC buffer, 0.02 M acetate-borate-cacodylate, was equally effective for both enzymes. As expected, buffers with metal binding capacity inhibited DNase I. 0.12 M succinate and citrate inhibited DNase II, 20% and 40%, respectively. In contrast to its activating effect on purified DNase II (13), 0.3 M acetate was slightly inhibitory to epidermal DNase II.

*Effect of Heat:* Epidermal homogenates were adjusted to pH 4.2 and 7.3, maintained at 61° C, and at regular intervals aliquots were removed and assayed at 37° C. The data indicate that at acid pH (Fig. 4, A) epidermal DNase II is heat stable at 61° C for 20 minutes or longer; it loses 40% of its activity after 10 minutes' heating at neutral pH (Fig. 4, B). DNase I is heat labile in both acid and neutral reactions.

*Effect of Beta Radiation:* The flanks of albino guinea pigs were given a single contact dose of 3000 rep $\beta$ rays from a $Sr^{90}$-$Y^{90}$ sealed source

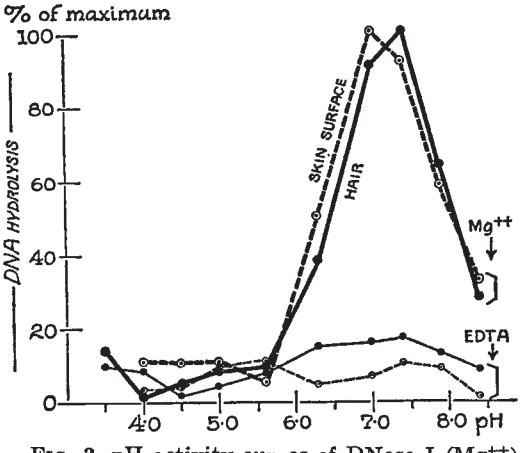


FIG. 3. pH activity curves of DNase I ($Mg^{++}$) and DNase II (EDTA) from saline extracts of guinea pig hair (solid line) and pooled washings from the clipped skin surface (broken line) (1). About 10% of the total DNase activity consists of heat stable DNase II.

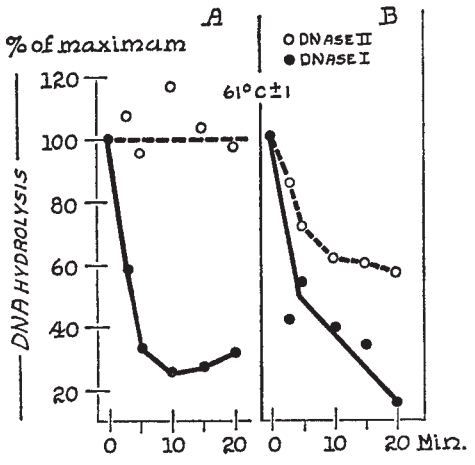


FIG. 4. A, heat stability of epidermal DNase II (broken line) and heat lability of DNase I (solid line) in homogenates kept at 61° C. and pH 4.2 to 4.8. B, increased heat sensitivities of DNase I and DNase II at pH 7.0 to 7.3. Each point is the average of data from 3 animals.

(14). Equal amounts of irradiated and adjacent, untreated epidermis were removed and washed as described (See METHODS). Each animal served as its own control. Sucrose-wash fractions and homogenates of washed tissue were assayed and the results, averages of 2 or 3 animals for each interval, are shown in Fig. 5. There is a progressive increase in extracellular DNase I activity and either no change or a decrease in DNase II activity. Expressed in units per mg protein (Fig. 5, A), extracellular DNase I (solid line) reaches a peak about 6 times the control by the tenth day. The intracellular DNase I activity (broken line) increases slightly by the sixth day. These increments are accompanied by proportionate increases in total DNase I activity. The decreases in DNase II at 1 and 10 days postirradiation may be statistically significant. In order to establish the validity of changes within 50% of the control values, additional animals are being tested. The numerically greater values obtained when the data were calculated in units per mg DNA may be an artifact reflecting the decrease in DNA content of irradiated epidermis beginning about the third day after exposure to 3000 rep $\beta$ (14).

### *Extracellular and Intracellular DNase I and DNase II Content of Albino Guinea Pig Epidermis*

About 40 to 50% of epidermal DNase I and II total activities are in the extracellular fraction (Table I, Column 8). Some enzyme may have been leached out from the anuclear dead cells of the *stratum corneum*. The ratio of DNase I to DNase II is slightly greater than 1. The differences in total activity (Table I) between washed epidermis and epidermis homogenized directly (8 animals) are not significant as determined by the "t" test. Using the conversion factor, 21 $\mu$g DNA-P released per $\Delta$ 260 unit (Fig. 1), the total specific activity of either DNase I or DNase II can be expressed as about 6.3 $\mu$g DNA-P per 30 minutes per mg protein (0.2 micromoles as acid-soluble deoxyribose); or, per mg DNA, the amount is 210 $\mu$g DNA-P (6.7 micromoles as deoxyribose).

### *DNase Activity in Other Organs*

When enzymatic activity per mg DNA (index of the degree of tissue cellularity or enzymatic activity per cell) is compared for various guinea pig tissues, pancreas has the highest level of DNase I and epidermis is next (Table II). Guinea pig hair contains equally high levels of DNase I readily extractable with saline. All of the visceral organs, excepting pancreas, have low DNase I levels, presumably due to the presence of a specific, heat labile DNase I inhibitor (13, 15). Since this enzyme is also heat-sensitive (Fig. 4) it is difficult to determine its true total activity in internal organs. To our knowledge, there have been no reports of a tissue inhibitor for DNase II.

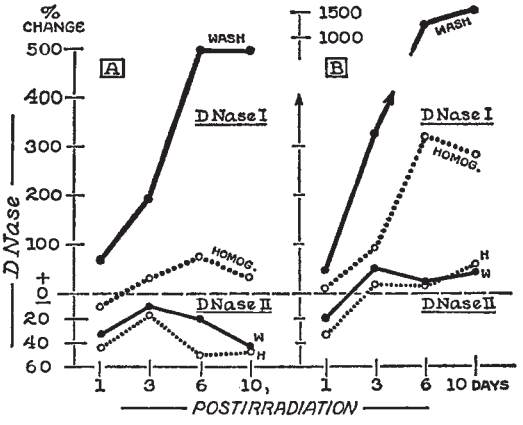


FIG. 5. Time-course curves showing difference in response of guinea pig epidermal DNase I and DNase II to a skin-surface dose of 3000 rep $\beta$ radiation; extracellular enzyme in 0.25 M sucrose-wash (solid line); intracellular enzyme in homogenates (broken line). A, % change calculated from units per mg protein; B, % change calculated from units per mg DNA. Each point is the average of data from 2 or 3 animals.

TABLE I

*Extracellular and intracellular DNase I and DNase II content of albino guinea pig epidermis*

| Number of Animals | Sucrose-Wash[a] | | Homogenate | | Total Activity | | % of Total Activity in Sucrose-Wash[b] | Total Activity DNase I / DNase II |
|---|---|---|---|---|---|---|---|---|
| | Units per mg protein | Units per mg DNA | Units per mg protein | Units per mg DNA | Units per mg protein | Units per mg DNA | | |
| **DNase I** | | | | | | | | |
| 9 | 0.15 ± 0.12[c] | 5.22 ± 0.66 | 0.22 ± 0.01 | 7.85 ± 0.60 | 0.39 ± 0.02 | 12.7 ± 1.03 | 40.0 ± 3.6 | 1.14 (0.97–2.40) |
| 8[d] | — | — | — | — | 0.32 ± 0.035 | 11.0 ± 0.83 | | 1.23 (0.98–2.19) |
| **DNase II** | | | | | | | | |
| 12 | 0.16 ± 0.01 | 5.30 ± 0.12 | 0.18 ± 0.014 | 5.88 ± 0.40 | 0.34 ± 0.013 | 11.2 ± 0.57 | 49.0 ± 3.1 | |
| 8 | — | — | — | — | 0.26 ± 0.02 | 9.76 ± 0.49 | | |

[a] Total units in sucrose-wash (see Methods, 2) divided by the mg protein or DNA in the washed tissue.

[b] The % of total activity present in the extracellular fraction and/or *st. corneum*.

[c] Standard error of the mean.

[d] The epidermis from these animals was homogenized directly, without prior washings with 0.25M sucrose. Average of DNA/mg protein for 14 animals, 31.2 ± 1.17 (S.E.).

TABLE II

*Comparison of specific activities of DNase I and DNase II of epidermis with those of other guinea pig organs*

| Tissue | Number of animals | DNase I | | DNase II | | Ratio DNase I/ DNase II |
|---|---|---|---|---|---|---|
| | | units/mg protein | units/mg DNA | units/mg protein | units/mg DNA | |
| Epidermis | 9 | 0.39 ± 0.02[a] | 12.7 ± 1.03 | 0.34 ± 0.013 | 11.2 ± 0.57 | 1.14 (0.97–2.40) |
| Hair | Pooled | 0.49[b] | — | 0.015 | — | 32.8 |
| Pancreas | 3 | 15.6 ± 2.25 | 362 ± 33.5 | 0.33 ± 0.03 | 6.0 ± 0.71 | 47.3 (40.0–59.8) |
| Liver | 4 | 0.042 ± 0.004 | 1.50 ± 0.075 | 0.092 ± 0.013 | 2.94 ± 0.42 | 0.46 (0.36–0.72) |
| Kidney | 4 | 0.11 ± 0.0 | 2.30 ± 0.22 | 0.17 ± 0.02 | 3.63 ± 0.42 | 0.65 (0.48–0.89) |
| Spleen | 4 | 0.06 ± 0.01 | 0.44 ± 0.06 | 0.36 ± 0.04 | 2.64 ± 0.19 | 0.17 (0.09–0.23) |
| Serum or plasma | Pooled or individual | <2 units/ml[c] | — | 0.0 | — | — |

[a] Standard error of the mean.

[b] Units per mg hair.

[c] The assay sensitivity is about one unit per ml of test solution. 0.1 aliquots of plasma contained ca. 20 μg heparin; this did not significantly inhibit DNase I. However, 100 μg heparin caused ca. 37% inhibition.

DNase II is present in higher concentration (per mg DNA) in the epidermis than in any of the other guinea pig tissues assayed (Table II). It could not be detected in fresh guinea pig plasma or serum and only trace amounts of DNase I could be found, even after 3 hours' incubation. Similar low levels were reported for human (16) and mouse (17) serum. Attempts to destroy any inhibitor(s) present in serum or plasma by subjecting the material to (a) repeated freezing-thawing, (b) heating to 55° C for 5 minutes, or (c) letting it stand at room temperature or 37° C for 24 hours, or at acid pH for 1 hour, resulted in no increase in DNase I activity.

### *DNases in Epidermis of Other Rodents*

The specific activities of DNase I and II were determined in epidermis removed from two Long-Evans (500 gm) and two albino Sprague-Dawley (350 gm) rats. The DNase II content of the former averaged 0.64 units per mg protein and 49.2 units per mg DNA; that of the latter, 0.77 units per mg protein and 40 units per mg DNA. These DNase II levels are about twice those of guinea pig epidermis (Table I). About 50% of rat DNase II activity was in the extracellular fraction or *stratum corneum*, or in both. The epidermis of both rat strains contained only trace amounts of DNase I.

## DISCUSSION

There is a gap in the biochemical literature concerning the nucleases in skin between the pioneering work of Wohlgemuth and Klopstock (18) in 1924 and recent reports (1, 2, 6, 19, 20). Because of the small amount of epidermal material available per mammal and the tedious procedures required to obtain it free of hair stubble, biochemists have focused their attention on the nucleases of the visceral organs and have neglected the epidermis,—this despite the fact that viewing the anuclear cells of the *stratum corneum* leads one to speculate whether or not the nucleases play a part in the disappearance of the nuclei. It has been suggested (19) that the nucleases play such a role during the keratinization process.

Data on localization of nucleases in skin were obtained primarily by histochemical methods (21, 22), which are indirect procedures difficult to execute and interpret. Employing the new substrate film method (22), Steigleder and Raab (23) found that DNase activity of human skin is strong in the sweat duct of normal skin and in the

entire *stratum corneum* of a psoriatic lesion. In rat epidermis (19) there are high concentrations of DNase II, but little or no DNase I. We have confirmed this, using two different rat strains. In contrast to rat and human epidermis, in which only DNase II activity can be detected (19), cavies appear to be unique in possessing high levels of both nucleases. It remains to be determined whether inability to detect DNase I in rat or human epidermis may be due to the presence of a specific inhibitor similar to the one in visceral organs (13, 15).

Of the five guinea pig organs assayed, epidermis contained the highest concentration of DNase II, 11.2 units per mg DNA, and the specific activity of DNase I was exceeded only by that of pancreas. About 45% of both DNase I and DNase II can be readily washed from excised epidermis, but washings of guinea pig skin surface or of hair (Fig. 3) contained high DNase I and only traces of DNase II. We can offer no explanation for this.

The question arises, does extracellular epidermal DNase originate primarily in this tissue? Enzymes which can be readily leached from excised epidermis by gentle washing could have come from dermal adnexae, from dead or dying cells of keratinized epidermis or from the interstitial compartment. In addition to low molecular weight nutrients, serum proteins have been found in normal guinea pig epidermis (24). The belief that extracellular DNase I and DNase II are primarily of epidermal origin is strengthened by the following observations: (a) After irradiation of guinea pig skin with a dose of 3000 rep $\beta$, the dermal adnexal structures atrophy or are completely destroyed by about the fifteenth day (14); enzyme present in the regenerating epidermis after this interval is probably of epidermal and/or humoral origin. Increased extracellular DNase I activity observed by the tenth day (Fig. 5) and later (25) could come only from the regenerating epidermis or the blood, or both. (b) The evidence is against a humoral origin, for DNase I is present in very low concentration while DNase II is absent from guinea pig plasma or serum (Table II). One would need to invoke a concentration mechanism or destruction of an inhibitor to regard extracellular epidermal DNase as coming from the blood. (c) Only traces of DNase activity can be detected in guinea pig dermis freed of epidermis. Similar results were obtained for rat dermis by Santoianni and Rothman (19).

Our investigations have revealed that about 45% of DNase I and DNase II, 75% of RNase (6) and 80% of inorganic pyrophosphatase (26) can be removed from excised epidermis by washing with physiological solutions.* These percentages indicate the proportion of total activity of the hydrolases in the interstitial compartment and/or *stratum corneum*. It would be of interest to determine the proportion of extracellular activity derived from each of these sources. Some of the extracellular DNase II may originate in sebaceous glands since its decrease postirradiation (Fig. 5) parallels the progressive atrophy of these glands.

With regard to intracellular DNase, histochemical data (23) on human epidermis show only slight activity in the region of the basal or malphigian layers. However, our biochemical data (Table I) on guinea pigs show that about 55% of total epidermal DNase I and DNase II are present within the cells; about 70% of this activity in homogenates of washed tissue is associated with cellular debris (25).

DNases I and II respond differently during the course of development of the radiation-induced lesion in the epidermis of guinea pigs (Fig. 5). Experiments in progress indicate that 24 hours postirradiation the increase in extracellular DNase I may not be significant; however, there is a significant increase by the third day. This is well before any grossly visible or microscopic evidence of injury. The histopathology of the lesion has been described (14). The increased extracellular DNase I reaches a peak about the tenth day postirradiation, when hyperkeratinization and hyperplasia are evident. At this interval, the increase can be detected on the skin surface of the living animal by means of the plastic cup technic (1, 25); DNase I can be leached from the dry, flaky crusts found above the injured area by the second week postirradiation. This increase in activity may be attributed to autolysis of dead or dying cells. As for epidermal DNase II activity, it remains unchanged or decreases during the 10 day interval following irradiation. It is of interest that in radio-sensitive tissues, such as spleen, thymus and bone marrow, this enzyme increases in activity, but DNase I remains unaltered. It is not known whether this difference in behavior of the epidermal DNases is specific

* On the basis of this information, it is clear that before attempting to determine the distribution of enzymes within cells of excised epidermis one should first wash this tissue with a physiological solution.

for this tissue or also characteristic of other radioresistant cells.

It is reasonable to assume (19) that nucleases play a role in degrading the nucleic acids of keratinizing epidermal cells. However, evidence for this is scanty and indirect, as follows: (a) nucleic acid depolymerases are present in high concentration in the horny layer of epidermis and in extracts of hair, (b) small amounts of breakdown products of the nucleic acids are found in keratinized tissues (27, 28), (c) DNase activity is decreased in epidermis of vitamin A treated rats, presumably correlated with a decrease in keratinized epidermis (19), and (d) DNase I activity in the epidermis of $\beta$-irradiated guinea pigs increases progressively and reaches a peak between the second and third weeks when hyperkeratinization and hyperplasia approach a maximum (25).

There remain unsolved problems regarding nuclease-mediated loss of nucleic acids in keratinizing tissue. First, in areas of hyperkeratinization, such as in irradiated epidermis undergoing dry desquamation, one would expect to find detectable increases in the breakdown products of nucleic acids. But we have not as yet detected any significant increase in these compounds, nor was there any evidence for the presence of the catabolic intermediates of thymine and uracil. Purine derivatives and pentoses have been isolated from normal human *stratum corneum* (27, 28), but the pyrimidine breakdown products of nucleic acids have not been detected in normal horny tissue (28). There is the possibility that in the differentiating epidermis these derivatives diffuse from the dying cells and are immediately re-utilized by the basal layer or absorbed into the circulation. Secondly, little is known regarding the fate (during keratinization) of the basic proteins (histones) associated with nucleic acids.

The biological function(s) of the nucleases is not known. Reports have appeared that DNase may play some part in DNA synthesis as well as in stimulating cell division. Crystalline DNase I and highly purified DNase II preparations increase the mitotic rate of many types of cells when supplied in physiological or low concentrations (1–10 $\gamma$ or less/ml) (29, 30). An exonuclease has been shown to increase the rate of *in vitro* DNA synthesis (31). DNase activity is at its highest level in rapidly dividing cells (32, 33).

Although the time spent in mitosis (mitotic duration) by normal epidermal cells is about one hour (34), the interval between divisions is unusually long for a proliferating tissue, ranging from 20 to 45 days. For the guinea pig, the renewal time (time taken for a cell to pass from the basal layer to the surface) of the squamous epithelium of hairy skin was estimated to be 82 days (35). If either DNase I or DNase II plays a part in cell division, then the relatively high concentration of these enzymes in guinea pig epidermis would lead one to expect a shorter generation time.

DeDuve (36) has conclusively demonstrated that in visceral organs there are present cytoplasmic particles (lysosomes) which contain on or within the confines of a lipoprotein membrane several acid hydrolases. These "bound" hydrolases can be released, with a concomitant increase in their activity, by treatment of homogenates of these organs with detergents or by other means. A similar release of hydrolases may also occur prior to and during cell autolysis (36). In spite of the slow renewal time of guinea pig epidermal cells, it is likely that the extracellular DNase I and DNase II activity in normal epidermis as well as the increased DNase I activity in irradiated tissue reflects a release and accumulation of these enzymes (as well as other hydrolases) from disintegrating cytoplasmic organelles as these cells differentiate and die. A brief report has appeared (37) indicating that there is histochemical evidence for the presence of lysosomes in mouse skin. Whether nucleases of the guinea pig epidermis are concentrated in lysosomes or lysosome-like particles remains to be determined.

## SUMMARY

1. In contrast to rat epidermis, which contains a high level of DNase II and no DNase I activity, guinea pig epidermis has both high DNase I and DNase II activities. The ratio is about 1.14. Saline extracts of guinea pig hair or washings from the skin surface contain high DNase I activity but only traces of DNase II. About 50% of guinea pig DNases I and II and rat DNase II can be removed by washing excised epidermis. This enzymatic activity is in the interstitial compartment or originates from the *stratum corneum*, or from both.

2. The DNase II content of guinea pig epidermis is higher than that of any of the visceral organs tested; its DNase I level is exceeded only by that of the pancreas.

3. Due to secondary changes caused by a skin

surface dose of 3,000 rep beta irradiation, there is a progressive increase in epidermal extracellular DNase I, while DNase II remains unchanged or decreases by the tenth day.

4. Evidence is presented that extracellular DNase I and DNase II activities originate primarily in the epidermis and not in the sebaceous glands or the blood.

5. The possible role of nucleases in the disappearance of nucleic acids during keratinization is discussed.

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